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# In vivo muscle regeneration and cross-sectional immunofluorescence

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An abbreviated version of this protocol was published in Life Science Alliance in Feb 2022

Muscle stem cell polarity requires QKI-mediated alternative splicing of Integrin Alpha-7 (Itga7)

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## Detailed protocol

Muscle injury via intramuscular injection of 50 ul of 10 uM cardiotoxin (CTX) in the tibialis anterior of the left leg only was performed on anesthetized mice. Carprofen analgesia was injected subcutaneously (20mg/kg) 30 minutes prior to CTX injection to alleviate discomfort associated with the procedure. Carprofen was also injected 24h and 48h following CTX injection to ensure continued comfort of the animal. After 3 weeks, mice were euthanized with $CO_2$ asphyxiation under isoflurane anesthesia. Tibialis Anterior muscle was isolated according to standard dissection techniques, please refer to the following resource for more details:

Template DNA-strand co-segregation and asymmetric cell division in skeletal muscle stem cells
Vasily Shinin [1], Barbara Gayraud-Morel, Shahragim Tajbakhsh
Affiliations
PMID: **19089364**
DOI: 10.1007/978-1-59745-060-7_19

**TA Cross-section and immunofluorescence protocol**

Materials Needed:

- 0.5% PFA in PBS (max 2 weeks old, stored at $4^0$C)
- 20% sucrose in ddH20 (w/v)
- 50:50 OCT:20% sucrose
  - Prepare 1 day in advance
- 15 ml falcon tubes
- aluminum foil, pen cap, and tape for cryomoulds
- OCT (VWR)
- Cryofreezing container (metal bowl over insulated chamber)
- Pasteur pipette or tissue probe
- Isopentane
- Liquid nitrogen
- Dry ice
- Superfrost coated slides (Fisher)
- PBS
- 0.1% triton, 0.1M glycine in PBS (for permeabilization)
- M.O.M Blocking reagent (Vector labs)
- 2% horse serum, 2% BSA in PBS
- DAPI mounting medium

Preparing the tissue:

1. Fill 15 mL tube with 4mL 0.5% PFA.
2. Dissect TA from mouse (as close to “tendon-to-tendon” as possible is ideal)
3. Place muscle in tube with 0.5% PFA and fix at $4^0$C for 2 hours.
4. Decant PFA from tubes into a beaker (in case muscle falls out)
5. Add 4 mL of the 20% sucrose solution to each tube
6. Equilibrate overnight at $4^0$C.

Preparing the cryomould:

1. Cut a 3x4 cm strip of aluminum foil and fold lengthwise to make a crease
2. Place bottom of pen cap down into one end of the foil in the crease, leaving half an inch of foil on one side of the cap and the remainder on the other.
3. Pinch the foil together around the pen cap, then fold the short end around the pen cap in one direction.
4. Then, fold the remainder of the foil around the pen cap in the other direction until it covers the circumference of the pen cap and cut off the extra foil.
5. Remove the pen cap from the foil and cut the open end of the foil to create an even opening. Label a piece of tape with the name of your sample and use it to hold the mold together.

Embedding the tissue:

1. Fill a Styrofoam box with liquid nitrogen and place in a chemical fume hood
2. Bring the insulated chamber and metal bowl into a chemical hood and using a scoop, fill the chamber with liquid nitrogen
3. Place the metal bowl above the liquid nitrogen and fill ~1/3 of the way with isopentane. Cover with foil and bring to the bench. Let the isopentane freeze for ~ 10 minutes
4. Retrieve you muscle samples and pour out the 20% sucrose. Replace with 4 mL of 50:50 OCT:20% sucrose.
5. Obtain a 15 mL falcon tube cap and fill with OCT
6. Grab the tendon of the TA muscle with tweezers and swirl around in the OCT until evenly and thoroughly coated with OCT.
7. Bring the TA to the cryomold and let it settle against the inner side of the mold, then release and let it slide down to the bottom
8. Fill the mold with OCT until the top of the tendon is submersed.
9. With tweezers, again pick up the tendon of the TA and slowly move it to the center of the cryomold such that the edges of the TA are not contacting the mold.
10. Using tweezers, pick up the cryomold and submerse it in the isopentane only up to the level of OCT. DO NOT FULLY SUBMERSE.
11. Hold the cryomold steadily in the isopentane until the OCT freezes and a bubble forms on top and freezes.
12. Move immediately to a Styrofoam box filled with dry ice.

At this point, the samples may be stored at -80$^0$C for future sectioning. Section 50uM thick slices and mount onto a SuperFrost microscope slide.

Immunofluorescence:

1. Rinse freshly mounted slides 3 times in PBS
2. Using a PAP pen, draw circles around sections according to how you wish to stain.
3. Permeabilize with 0.1% triton 0.1 M glycine in PBS for 10-12 minutes at RT
4. Wash 3x 5 minutes with 1x PBS
5. Block with M.O.M blocking reagent (1 drop in 1.25 mL PBS) for minimum 1 hour at RT
6. Incubate with primary antibodies overnight at 4$^0$C in humidity chamber (ie. Slide box with wet paper towel in it).
    a. For concentrated Pax7 monoclonal mouse antibody (DSHB), use 1:100 diluted in 2% HS 2% BSA in PBS
    b. For laminin antibody (Sigma L9393), use 1:100 diluted in 2% HS 2% BSA in PBS
7. The following morning, wash 3x 10 minutes in PBS on a shaker.
8. Use AlexaFluor secondary antibodies at a concentration of 1:400 diluted in 2% HS 2% BSA in PBS. Incubate in the dark at RT for 45 minutes.
9. Wash 3x 10 minutes in 1x PBS on a shaker
10. Mount slides with DAPI mounting medium and seal coverslip with nail polish.

To visualize injury induction, TA muscle can be isolated and at 3 weeks following injury. Myofibers will be delineated by laminin stain and should contain centrally-localized myonuclei if the TA was successfully injured. Earlier stages of muscle regeneration can also be visualized at earlier time points.

**How to cite:**(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Dominici, C. and Richard, S. (2022). In vivo muscle regeneration and cross-sectional immunofluorescence. Bio-protocol Preprint. bio-protocol.org/prep1769.
2. Dominici, C. and Richard, S.(2022). Muscle stem cell polarity requires QKI-mediated alternative splicing of Integrin Alpha-7 (Itga7). Life Science Alliance 5(5). DOI: 10.26508/lsa.202101192